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Osteonectin Interacts with Human Nebulin C-terminus in Skeletal Muscle

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Nebulin is a giant actin binding protein (600~900 kDa) which is specific to skeletal muscle. This protein is known to regulate thin filaments length in sarcomere as a molecular template. The C-terminus of nebulin is located in the Z-disc of muscle sarcomere and is bound to other proteins such like myopalladin, titin, archvillin, and desmin. The N-terminus of nebulin binds to tropomodulin at the pointed ends of the thin filaments. In recent research, nebulin not only found in brain but also expressed in heart, stomach, and liver. So, the roles of nebulin in non-muscle tissue have been studied. However, lack of information or studies on nebulin binding proteins and nebulin function in brain are available so far. Therefore, the current study have investigated a novel binding partner of Nebulin C-terminus by using yeast two-hybrid screening with human brain cDNA library. Nebulin C-terminus, containing simple repeats, serine rich and SH3 domain, interacts with osteonectin C-terminal region. The specific interaction of nebulin and osteonectin were confirmed in vitro by using GST pull-down assay and reconfirmed *in vivo* by using transfected COS-7 cells with EGFP-tagged nebulin and DsRed-tagged osteonectin. Consequently, this study identified SH3 domain in nebulin C-terminus specifically binds to extracellular Ca-binding (EC) domain in osteonectin. Also, nebulin C-terminus fusion protein colocalized with osteonectin EC domain fusion protein in transfected COS-7 cells. The current study found the interaction between nebulin and osteonectin in human brain for the first time and suggested the nebulin in brain may be associated with osteonectin, as a regulator of cell cycle progression and mitosis.

Key Words: Nebulin, Osteonectin, Yeast two-hybrid, GST pull-down assay, COS-7

INTRODUCTION

Nebulin is a giant actin-binding protein with size variants from 600 to 900 kDa in various skeletal muscles (Wang and Wright, 1988) and single molecules of nebulin span the entire length of the mature thin filament (Trombitas et al., 2001; Wang et al., 1996). Nebulin is encoded by a single gene that exhibits extensive alternative splicing, which produces isoforms (McElhinny et al., 2003). Alternative splicing in the central and C-terminal regions results in the expression of various nebulin isoforms in different skeletal muscle types, developmental stages and species (Hu et al., 1986; Labeit and Kolmerer, 1995; Locker and Wild, 1986; Millevoi et al., 1998; Pelin et al., 1999). The molecular size of nebulin isoforms correlates with thin filament length variations in different skeletal muscle types, supporting the hypothesis that nebulin functions as a molecular template to specify the lengths of the thin filaments (Kruger et al., 1991; Labeit et al., 1991; Trombitas et al., 2001). Analysis of the nebulin cDNA sequence also supports its role as a thin filament template (Labeit and Kolmerer, 1995; Wang et al., 1996; Labeit et al., 1991). Human nebulin cDNAs have revealed an extensively modular domain structure that appears to be ideally suited for dictating thin filament

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architecture (Labeit and Kolmerer, 1995; Wang and Wright, 1988). The N-terminus of nebulin contains a unique 8 kDa segment of unknown function and modules M1-M8. The modules M1-M3 interact with the thin filament pointed end capping protein, tropomodulin (McElhinny et al., 2001). It has been proposed that the interaction of nebulin with tropomodulin may contribute to the regulation of thin filament lengths in muscle that supports nebulin's proposed role as a template molecule. The central region of nebulin is made up of 185 repeats that are each about 35 amino acid residues in length; these modular repeats are referred to as M1-M185 and constitute 97% of the molecule (Labeit and Kolmere, 1995). The central 154 modules (M9-M162) make up 22 super-repeats of 7 modules. Each super repeats interacts with a troponin-tropomyosin regulatory complex of the thin filament (Chen et al., 1993; Holmes et al., 1990; Jin and Wang, 1991; Pfuhl et al., 1996; Wang et al., 1996). The segment comprising repeats M163-M170 links nebulin's super repeat region to the C-terminal region modules M171-M185, which are located close to the periphery of the Z-line. Modules M160-M170 binds to the intermediating filament protein desmin, suggesting that they may function in maintaining the lateral registry of adjacent myofibrils (Bang et al., 2002). The two C-terminal domains, the serine rich domain and the SH3 domain, are highly conserved between species, and they are expressed in the Z-disc of all types of skeletal muscle early in developing myofibrils (Lin et al., 1994; Politou et al., 1998; Millevoi et al., 1998). The two domains have been predicted to be involved in the dynamics of myofibrillar assembly and disassembly. The nebulin extreme C-terminal end contains a SH3 domain and multiple phosphorylation motifs, suggest that nebulin is involved in signaling events within the Z-line (Labeit and Kolmerer, 1995) (Fig. 1A). In this regard, nebulin's SH3 domain binds to myopalladin, an interaction that appears to be critical for myofibril assembly and/or stability (Bang et al., 2001a; Clark, 2002).

Recently study reports that the expression of nebulin mRNA transcripts in brain, heart, stomach and liver of 15-day-old chicken embryo and in brain of human. Eleven different human nebulin isoforms have been identified in the Z-disc region modules M174-M182, each consists of 31-residue motif. Four brain isoforms and nine types of skeletal isoforms are also detected (Joo et al., 2004). However the interacting partner and the function of nebulin in brain is not revealed. The interaction between nebulin C-terminus and osteonectin was characterized using the yeast two-hybrid system, GST pull down assay and immunofluorescence microscopy experiments in COS-7 cell.

MATERIALS AND METHODS

1. Yeast two-hybrid interaction study

1) Bait plasmid preparation

Human brain Large Insert cDNA library (Clontech) was used as template for Neb C PCR amplification for 35 cycles by using forward primer HNC I and reverse primer HNeSH3 II. PCRs were hot-started by adding all reagents except DNA polymerase, heating to 95℃ for 10 min then holding at 80℃ for 45 min (Amaravadi & King, 1994). Enzyme (Ex Taq polymerase, Takara) was added to mixture and amplification was performed 35 cycles (95℃ for 1 min, 56℃ for 30 sec and 72℃ for min). The PCR products were cloned using the pGEM-T vector system (Promega). The ligated plasmid was digested with *EcoR* I and *Sal* I and cloned into the pGBKT7 (Clontech) vector.

2) Library screening

The yeast two-hybrid screen was carried out using a GAL4-based yeast two-hybrid system (MATCHMAKER Two-Hybrid System 3; Clontech) and was performed as recommended by the manufacturer (Clontech). Bait constructs were transformed into yeast strain AH109 (Meta) and the transformants were plated on dropout medium lacking tryptophan (SD/-Trp) because the pGBKT7 vector had a selectable *TRP1* marker. The pretransformed Human Brain Matchmaker cDNA Library, which is a human brain cDNA library that has been cloned into a pACT2 vector with a selectable *LEU2* marker for expression of fusions with the GAL4 activation domain (AD) (aa, 768~881 of GAL4), and which has been pretransformed into yeast strain Y187 (Mata), was purchased from Clontech.

The two transformant cultures were mated to each other and initially plated on dropout medium lacking leucine and tryptophan (SD/-Leu/-Trp) to select for both the bait and

prey plasmids. In this two-hybrid system, the GAL4 BD binds to the GAL upstream activating sequence and, if the fusion proteins interact, the AD is brought into proximity with the promoters of four reporter genes (*HIS3*, *ADE2*, *MEL1*, and *lacZ*), thereby activating transcription and permitting growth on selection media (SD/-Ade/-His) and the expression of α-galactosidase (*MEL1* product) and β-galactosidase (*lacZ* product). The cotransformants were then plated on dropout medium lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp) to select for colonies that expressed interacting proteins. Positive interactions were confirmed by cell growth on SD/-Ade/-His/-Leu/-Trp medium and by α-galactosidase assays.

3) Sequencing analysis

The identified clones were purified by using QIAprep Spin Miniprep Kit (Qiagen) and sequenced using Thermo Sequenase Cycle Sequencing Kit (USB) and LI-COR 4200 (LI-COR) as described by the manufacturer. Sequences were identified using Blast search of NCBI.

4) Deletion construction of Neb C and osteonectin

In order to investigation of potential nebulin and osteonectin interactions, each fragment was amplified from cloned Neb C and osteonectin plasmid by PCR. Amplified with Neb C PCR fragments were inserted into pGBKT7 vector to obtain GAL4-DNA BD fusion and osteonectin PCR fragments were inserted into pGADT7 vector to obtain GAL4-DNA AD fusion.

2. *In vitro* transcription/translation and GST pull-down assay

1) *In vitro* transcription and translation

In vitro T7-driven transcription and translation of nebulin C-terminus was done with the TnT® T7 Quick Coupled Transcription/Translation System (Promega) with Transcend Biotin-Lysyl-tRNA (Promega). A similar reaction using exception of DNA was also used as a negative control. To label the peptides, the reactions were performed using the Transcend Non-Radioactive Translation Detection System as described by the manufacturer (Promega). The size of in vitro transcription and translation proteins were determined by 12% SDS-PAGE and western blot analysis.

2) GST fusion protein preparation and expression

pACT2-osteonectin was digested with *Sal* I/*Xho* I and inserted into expression vector, pGEX-4T-3 (Amersham Biosciences) to obtain GST fusion for GST pull-down assay. Overexpression of GST-osteonectin and GST alone (as a control) were induced with isopropyl-β-d-thiogalactopyranoside (IPTG) in *E. coli* strain BL21 (Amersham Biosciences). The cells were collected by centrifugation and lysed by sonication. Fusion proteins were purified from the lysates by affinity chromatography on reduced glutathione Sepharose. To confirm the presence of GST-fusion protein was determined by 12% SDS-PAGE and western blot analysis.

3) GST pull-down assay

The purified GST fusion proteins were incubated for 1 h at room temperature with the resin to immobilize the fusion protein; glutathione-Sepharose 4B beads (Amersham Biosciences) for GST. The beads were washed three times with PBS by 10 folds of bed volume and the GST fusion protein was resuspended in interaction buffer (PBS, 1% Triton X-100, 1% BSA). For binding experiments, 5 μl of biotin-labeled TnT® protein extract was incubated with fusion protein (50 μl of beads, 50% slurry) in the interaction buffer for 90 min at room temperature with shaking. Beads were washed three times with wash buffer, PBS containing 1% Triton X-100. Biotin-labeled proteins eluted in sample buffer (0.15 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) were separated by 12% SDS-PAGE and western blot analysis.

3. Fluorescence microscopy

1) Plasmid preparation

To generate the EGFP-tagged and DsRed-tagged expression vectors, pGBKT7 Neb C was digested with *EcoR* I/*Sal* I and inserted into mammalian expression vector, pEGFP-C2. And osteonectin was amplified by PCR using appropriate primers containing *Xho* I/*Sal* I. The reaction was performed with Advantage-HF 2 PCR kit (BD Biosciences Clontech). Generated DNA product was digested with *Xho* I and *Sal* I, and inserted into pDsRed1-C1 vector (Clontech). This plasmid was purified with QIAGEN plasmid midiprep kit (Qiagen).

2) Cell culture and transfection

COS-7 cell, a monkey kidney fibroblast, was purchased from the Korean Cell Line Bank (KCLB # 21651). The cells were maintained in DMEM supplemented with 10% fetal bovine serum. COS-7 cells were transfected with EGFP-Neb C constructs and DsRed-osteonectin constructs using the FuGENE 6 Transfection Reagent (Roche) according to the manufacture's instructions. The cells were in-

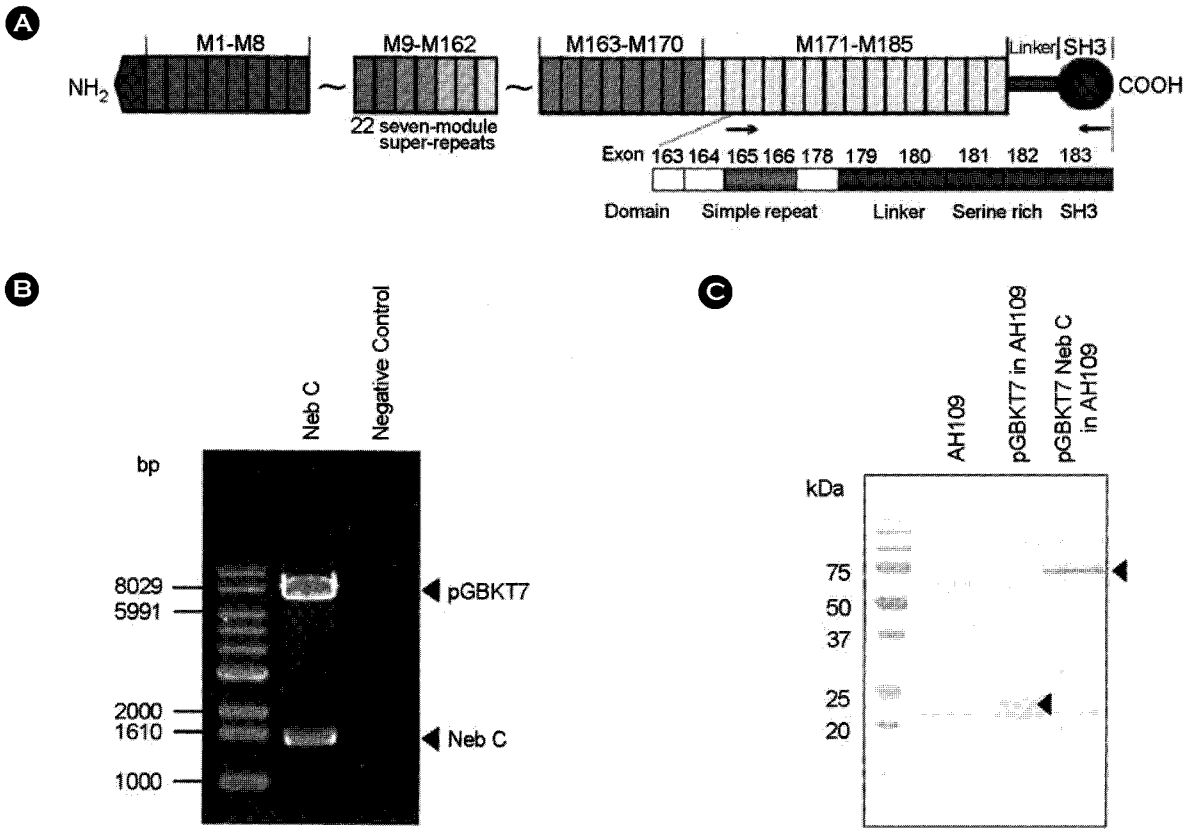

Fig. 1. Preparation of nebulin C-terminal fragment for bait plasmid construction and expression of the GAL4 BD-Neb C fusion protein in yeast (AH109). **A.** Nebulin fragment was amplified from human brain Large-insert cDNA library using nebulin-specific primers (HNCI and hNeSH3II). **B.** Cloning of the GAL4 BD-Neb C DNA. Nebulin C-terminal cDNA inserted into pGBKT7 vector. **C.** Expression of the GAL4 BD-Neb C fusion protein in yeast. Protein samples were resolved by electrophoresis on a 12% SDS-PAGE gel. Proteins were electroblotted from the gel to a nitrocellulose membrane, which was probed with a C-Myc monoclonal antibody, followed by an AP-conjugate of goat anti-mouse IgG antibody.

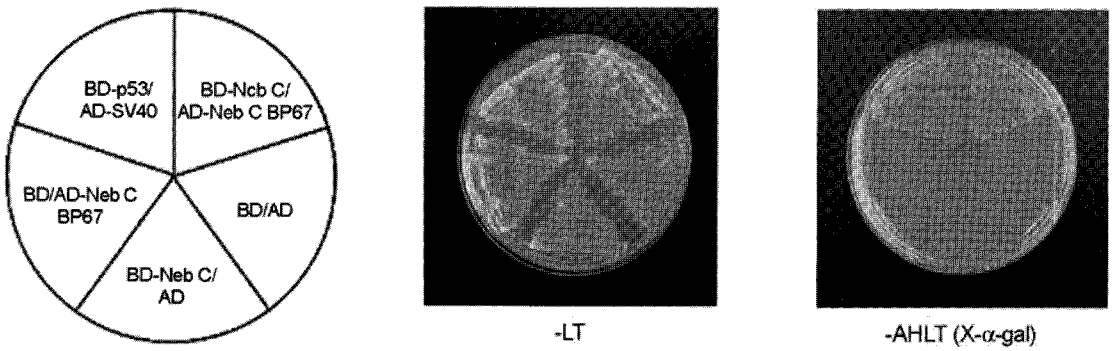

Fig. 2. Yeast two-hybrid analysis of the interaction of human NebC and osteonectin. Growth of transformed yeast on selective plates. Yeast strain AH109 was cotransformed with pGBKT7-Neb C and pACT-Neb C BP67 incorporating the indicated constructs (Left). Plasmids pGBKT7-53 and pACT2-SV40 large T antigen were transformed as a positive control because p53 protein is known to interact with SV40 large T-antigen. Yeast two-hybrid analysis followed by X-gal assay indicated that Neb C specifically interacted with Neb C BP67.

cubated under 5% CO_2 atmosphere at 37℃ for approximately 48 h before processing. After fixation, cells were observed on a fluorescence microscope BX50 (Olympus) with 40X oil immersion objective. Images were recoded using a digital camera DP70 (Olympus) connected to DP70-BSW Version 01.02 software.

RESULTS

1. Molecular cloning of the human nebulin C-terminus cDNA

About 1.5 kb of human nebulin C-terminus (Neb C) was amplified by PCR using Human Brain Large Insert cDNA library (Clontech) as template and performed PCR using appropriate primers (HNC I and hNeSH3 II) containing *EcoR* I/*Sal* I restriction enzyme site designed from the human nebulin cDNA sequence (accession No. X83957). The Neb C sequence was identical to the skeletal muscle cDNA as determined by Blast alignment of National Center for Biotechnology Information. It contains two simple repeat (exons 165 and 166), linker, serine-rich and SH3 domain (Fig. 1A).

2. Human nebulin C-terminus binds to osteonectin in yeast two-hybrid experiment

To identify potential interaction partner of the nebulin, a human brain cDNA library was screened using the yeast two-hybrid technique and the C-terminal fragment of the nebulin cDNA as bait. The pGBKT7-Neb C vector was transformed into yeast strain AH109 (Fig. 1B). The pGBKT7-Neb C expression construct yielded a fusion protein of the expected molecular size (about 70.5 kDa), indicating that the GAL4 BD-Neb C fusion was suitable for use as bait in our two-hybrid protein interaction screening (Fig. 1C). Human brain cDNA library cloned into pACT2 was screened. Out of 3.8×10^7 transformants, it was obtained >1,000 colonies that were positive for the expression of the selection markers (*HIS3*, *ADE2*, and *MEL1*). As shown in Fig. 2, yeast cells transformed with both pGBKT7-Neb C and pACT2-Neb C BP67 grew on dropout medium lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp/X-α-gal), as a positive control (p53, SV40), while the other double transformants did not.

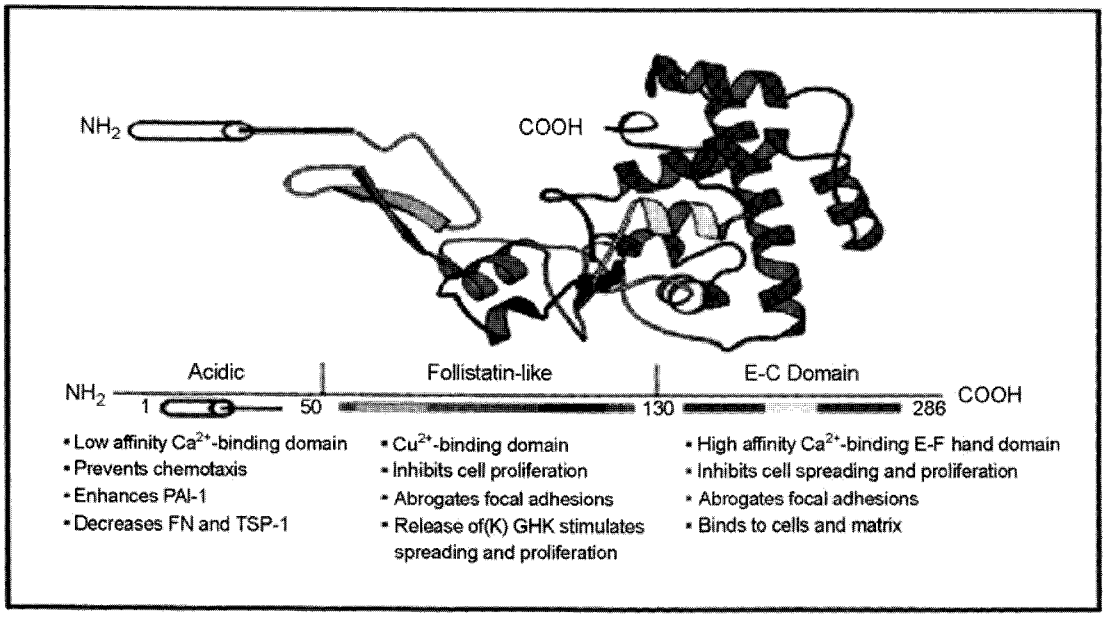

Fig. 3. Modular structure of human osteonectin. The ribbon diagram derived from crystallographic data indicates three structural modules. The follistatin-like domain, aa 53~137, is shown in red except for peptide 2.1, aa 55~74, and the (K) GHK angiogenic peptide, aa 114~130, which are shown in green and black, respectively. The EC-module aa 138~286 is shown in blue except for peptide 4.2, aa 255~274, which is shown in yellow. Adapted from Hohenester et al., 1997 and the Brookhaven Protein database, accession number 1BM0. PAI, plasminogen activator inhibitor; FN, fibronectin; TSP, thrombospondin.

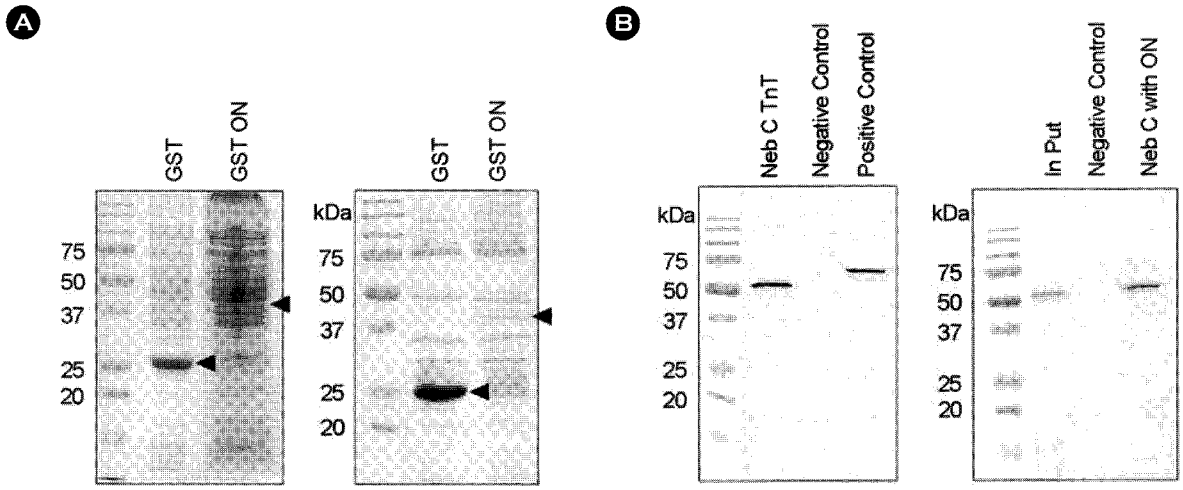

Fig. 4. SDS-PAGE and western blot analysis of GST osteonectin (ON) and GST pull-down assay. Bacterially Expressed, GST and GST osteonectin fusion proteins were fractionated on 12% SDS/PAGE gel. Recombinant proteins were electroblotted from the gel to a nitrocellulose membrane, incubated with anti-GST antibody, followed by an AP-conjugate of rabbit anti-goat IgG antibody. One microgram of pGBKT7 Neb C plasmid was used in the TNT Quick coupled transcription/translation system and detected by the Transcend non-radioactive translation detection system. *In vitro*-translated Neb C (Input) bound to glutathione-Sepharose 4B beads in the presence of GST-osteonectin fusion protein.

3. Sequence analysis of Neb C BP67

DNA sequencing revealed that Neb C binding protein (Neb C BP67) is a osteonectin. It is located on chromosome 5q31.3~q32. The full length cDNA sequence of osteonectin was searched by blast of NCBI and identified a 3.2 kb full-length cDNA for osteonectin (accession no. NM_003118), which encodes a protein of 303 amino acids with a predicted molecular mass of about 32 kDa. Osteonectin containing Follistatin (FS)-like domain and Extracellular (EC) domain is binding with nebulin (Fig. 3).

4. GST pull-down assay of human Neb C and osteonectin

To characterize the interaction of Neb C and osteonectin *in vitro*. The molecular weight of the fusion protein was about 26 kDa (GST) and about 44.5 kDa (GST-osteonectin), respectively (Fig. 4A). *In vitro* transcription/translation, Neb C gene was cloned into the pGBKT7 expression vector and sequenced before expression. The proteins were migrated at an approximately size of 57 kDa. GST-osteonectin or GST alone was incubated with the *in vitro* translation products of Neb C and glutathione-Sepharose 4B beads, the Neb C bound to GST-osteonectin but not to control GST (Fig. 4B).

5. Identification of the minimal region required for Neb C and osteonectin interaction

To further narrow down the osteonectin-binding site within nebulin, Neb C was generated five deletion constructs (Fig. 5A). Truncated Neb C fragments were amplified from Neb C cloned pGBKT7 with appropriate primers and verified with DNA sequencing. Simple domain contains exon 163 to 178 that encode 2 modules of simple-repeat (C1), simple-repeat and linker domain contain exon 179~180 (C2), linker and serine-rich domain contain exon 179~182 (C3), serine-rich domain contains exon 181 and 182 (C4), and SH3 domain is exon 183 (C5). To identify the region of osteonectin that actually involved in binding to Neb C, the osteonectin was generated two deletion construncts (Fig. 5B). Yeast cells transformed with both pGBKT7-Neb C and pGADT7-ON grew on the SD/-Ade/-His/-Leu/-Trp/ X-α-gal plate, as a positive control (p53, SV40), while the other double transformants did not. As a result, nebulin SH3 domain is binding to osteonectin C1 region (Fig. 5C).

6. Colocalization of Neb C and osteonectin in COS-7 cell

To determine the subcellular localization and colocalization of EGFP-Neb C with DsRed-osteonectin fusion proteins, COS-7 cells were transfected with each fusion con-

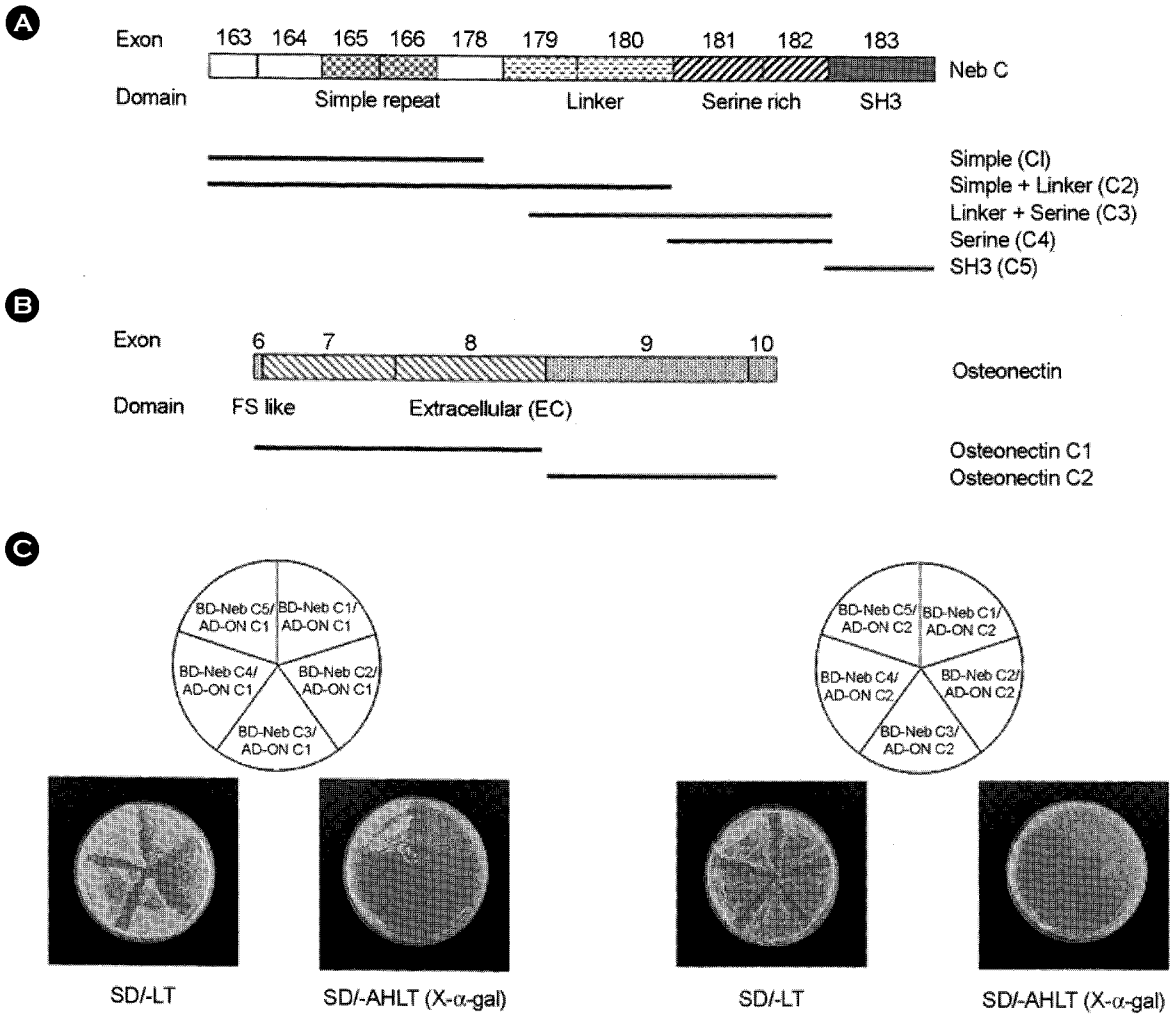

Fig. 5. Identification of minimal binding region for nebuin and osteonectin interaction. **A.** The nebulin deletion constructs as baits for osteonectin. Major protein domains of nebulin C-terminal region are shown. Chimeric constructs were generated within the pGBKT7 backbones (C-terminally fused) and contain various fragments of nebulin. **B.** osteonectin deletion constructs as preys for nebulin. Major protein domains of osteonectin are shown. Chimeric constructs were generated within the pGADT7 backbones (C-terminally fused) and contain various fragments of osteonectin. **C.** Identification of minimal binding region by the yeast two-hybrid system. pGBKT7-Neb C fragmnets were cotransformed with pGADT7-osteonectin fragmnets into AH109. Transformed AH109 cells containing both plasmids were streaked out on plates lacking leucine and tryptophan (SD/-LT) or plastes lacking adenine, histidine, leucine, and typtophsn (SD/-AHLT/ X-α-gal). Only nebulin's SH3 domain (Neb C5) interact with osteonectin C1 (ON C1).

struct separately or cotransfected with both fusion constructs together. As shown in Fig. 6, the EGFP-Neb C fusion protein was localized exclusively in the cytoplasm of cotransfected COS-7 cells. Especially, it was observed punctate around nucleus. On the other hand, the DsRed-osteonectin protein was concentrated around in the nuclei of cotransfected COS-7 cells. The control EGFP vector and DsRed vector were localized throughout the cell. In COS-7 cells, cotransfected with EGFP-Neb C and DsRed-ON were distributed almost peripheral cytoplasm and around nuclei (Fig. 6).

DISCUSSION

This study is the first report on the identification of a novel binding partner of nebulin C-terminus in human brain cDNA library. Nebulin C-terminus, contains simple repeat, serine-rich and SH3 domain, interacts with osteonectin with yeast two-hybrid screening. This finding is demonstrated with GST pull-down assay and immunofluorescence microscopy studies in COS-7 cells. *In vitro* transcription and translation Neb C protein bound to GST-osteonectin fusion protein. In addition, EGFP-Neb C and DsRed-osteonectin

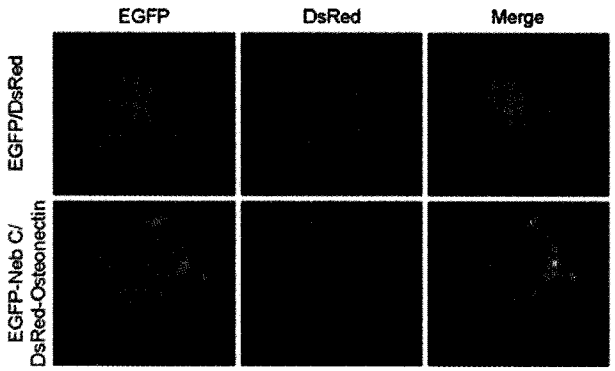

Fig. 6. Colocalization of Neb C and Osteonectin in COS-7 cells. COS-7 cells were transiently transfected with EGFP, DsRed control vector or with the fusion construct, EGFP-Neb C, DsRed-osteonectin. After 48 hrs, living cells were fixed and observed using a fluorescence microscope linked to a digital camera DP70 (Olympus). All three panels of a row have the same field of view with ×400 objective lens. Nucleus was stained with DAPI.

colocalize with in COS-7 cells.

Osteonectin, also termed SPARC (Secreted Protein, Acidic and Rich in Cysteine), is a 32 kDa calcium binding matricellular glycoprotein secreted by many different types of cells (Bornstein, 1995). This protein is associated with cellular populations undergoing migration, proliferation, and differentiation (Schwarzbauer and Spencer, 1993). Although the mechanisms by which it exerts its effects have not been fully elucidated, osteonectin is generally characterized as a secreted counter adhesive protein that modulates the interaction of cells with components of the extracellular matrix, in part through disassembly of focal adhesion complexes (Sage et al., 1989; Murphy-Ullrich et al., 1995). The human osteonectin consists of 286 residues divided into three distinct domains (Fig. 3). The N-terminal domain (residues 1~52 after a 17-amino-acid signal sequence) is an acidic region rich in Asp and Glu. Domain I binds several calcium ions with low affinity (Maurer et al., 1992) and interacts with hydroxyapatite (Romberg et al., 1985). Domain II is a Cys-rich, follistatin-like (FS) domain (residues 53~137), in which all the Cys residues are disulfide-bonded, and with an N-linked complex carbohydrate at Asn 99. The FS domain is homologous to follistatin and Kazal-type protease inhibitors (Patthy 1991). It also contains two copper binding sites (Vernon and Sage, 1989), one of which, the sequence KGHK (residues 119-122), stimulates cell proliferation and angiogenesis (Lane et al., 1994). Glycosylation of osteonectin at Asn 99 of the FS domain is another example of a posttranslational modification that alters the function of the protein. Domain III (the extracellular calcium binding domain (EC domain), residues 138~286) is largely α-helical and contains a canonical pair of EF-hands, with high affinity calcium binding sites. Domain III was predominantly α-helical and contained a binding site for collagen IV (Mayer et al., 1991). The EF-hand pair interacts tightly with an amphiphilic N-terminal helix and has been defined as a novel calcium binding module (Hohenester et al., 1996). This domain also has a binding epitope of moderate affinity for collagen Types I and IV (Sasaki et al., 1998). An endogenous protease cleavage site is located at a Leu-Leu bond in position 197/198 in the α-helical region (Mann et al., 1987; Mayer et al., 1991).

The novel recent study showed that osteonectin was detected in the nuclear matrix of cells by immunocytochemistry, both in chick embryonic Day E2 and adult bovine aortic endothelial cells pretreated to remove soluble proteins and chromatin; embryonic chicken cells, E2 and E10. Moreover, osteonectin was expressed at high levels in the cytoplasm of M-phase cells at metaphase and anaphase. These findings were confirmed by the use of several anti-SPARC antibodies and by immunoblotting of isolated nuclei and cytoplasmic fractions. Furthermore, it was shown that osteonectin was taken up and translocated to the nuclei of cultured embryonic chicken cells (Gooden et al., 1999). That is to say, (a) Osteonectin is a component of the nuclear matrix during interphase; (b) expression of intracellular osteonectin is correlated with specific stages of the cell cycle, during metaphase and anaphase; and cells of late telophase were devoid of osteonectin immunoreactivity; and (c) ganglion cell neurons, which are postmitotic, showed cytoplasmic staining but no nuclear labeling (Yan et al., 1998).

Recently study shows a function for osteonectin in enhancing RGC (retinal ganglion cells) regeneration, as demonstrated by both neuronal survival and neurite outgrowth from axotomized neurons. Osteonectin is widely expressed in the brain during development, with some expression maintained in the adult (Mendis and Brown, 1994; Mendis et al., 1995, 1996), and is upregulated at sites

of injury, including the CNS (Mendis et al., 2000; Bradshaw and Sage, 2001). In summary, osteonectin plays a role in neural regeneration and this may be exploited for therapeutic CNS repair.

Consequently, this study identified that osteonectin is interacting with nebulin C-terminus and colocalizing with nebulin in COS-7 cell. Furthermore, it is provided data for the potential binding region between two proteins. This data may predict that unknown function of nebulin in brain is associated with osteonectin. However, further studies will be necessary to clarify the role of the nebulin and osteonectin in brain.

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